REMARKS

I. Status of Claims

Claims 1-28 are pending in this application.

In the present Amendment, claims 9 and 16 have been amended to more appropriately claim the invention. Support for the amendments can be found in the specification, paragraphs [0149]-[0155]. Applicants have not introduced any new matter by the amendment, nor does the amendment raise new issues or necessitate the undertaking of any additional search of the art by the Office.

II. Obviousness-Type Double Patenting Rejection

The Examiner provisionally rejects claims 1-8 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claim 8 of co-pending Application No. 10/715,358 ("the '358 application"). Office Action, page 3.

Solely to advance prosecution, Applicants have filed a Terminal Disclaimer. Accordingly, Applicants respectfully request that the Examiner withdraw this rejection.

III. Rejection under 35 U.S.C. § 112, First Paragraph, Written Description

The Examiner rejects claims 1-28 under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. Office Action, page 4. Specifically, the Examiner alleges that the new provisos recited in claims 1 and 9, *i.e.*, provisos (2) and (3), are not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors, at the time the application

was filed, had possession of the claimed invention. *Id.* Applicants respectfully disagree with the Examiner and traverse this rejection for at least the following reason.

The compounds of formula (I), as described in the originally-filed specification, include the compounds that were "provisoed out" in provisos (2) and (3), *i.e.*, 3-{4-(3,4,5-trimethoxyanilinocarbonyl)-3-oxo-2,3-dihydropyridazine-6-yl}-2-phenyl-pyrazolo[1,5-a]pyridine; 3-{4-(N-ethoxycarbonylmethyl)-carbamoyl-3-oxo-2,3-dihydro-pyridazine-6-yl}-2-phenyl-pyrazolo[1,5-a]pyridine; 3-{4-(N-carboxymethyl)-carbamoyl-3-oxo-2,3-dihydro-pyridazine-6-yl}-2-phenyl-pyrazolo[1,5-a]pyridine; 6-(4-cyanophenyl)-4[(4-carboxybutyl)-aminocarbonyl]-2H-pyridazin-3-one; 6-(4-methoxyphenyl)-4-methylcarbamoyl-2H-pyridazin-3-one, and compounds of formula (I) wherein when A is $NHCOCH(CH_3)_2$, Ar is unsubstituted or at least monosubstituted bicyclic heteroaryl.

A limitation excluding species of a genus is sufficiently supported by an original specification that taught the entire genus, because the "specification, having described the whole, necessarily described the part remaining," as well as the part being provisoed out. *See In re Johnson*, 194 USPQ 187, 196 (CCPA 1977). "It is for the inventor to decide what *bounds* of protection he will seek." *Id.* (emphasis in original). Accordingly, Applicants respectfully request this rejection be withdrawn.

IV. Rejection under 35 U.S.C. § 112, First Paragraph, Enablement

The Examiner rejects claims 9-25 under 35 U.S.C. § 112, first paragraph, for lack of enablement. Office Action, pages 4-9.

A. Claims 9-16

The Examiner alleges that because claims 9-16 cover the inhibition of CDK2 "in any patient the claims are not enabled. *Id.* at page 9 (emphasis in original). Applicants respectfully disagree. However, solely to advance prosecution, Applicants have amended claims 9 and 16. Applicants respectfully submit that claims 9-16 as amended are fully enabled by the specification for at least the following reasons.

"The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation." M.P.E.P. § 2164.01. Further, "if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate." *Id.* § 2164.02.

As shown in a recent article, Payton et al., "Discovery and Evaluation of Dual CDK1 and CDK2 Inhibitors," Cancer Research, 66(8), 2006, pages 4299-4308 ("Cancer Research 2006 article"), a small-molecule CDK inhibitor, which inhibits CDK2 *in vitro* (*see* Table 1 on page 4302), also inhibits CDK2 *in vivo* (*see* pages 4305-4306). A copy of this Cancer Research 2006 article is attached for the Examiner's convenience.

Here, the originally-filed specification shows, under "CDK2/Cyclin E Flashplate Assay: 96-well format" in paragraphs [0301]-[0303], that the inventive compounds have potency of inhibiting the kinase CDK2 as indicated by the IC_{50} values. The Examiner has failed to establish that this *in vitro* data is not recognized as correlating to *in vivo* inhibitory potency of CDK2. Therefore, contrary to the Examiner's allegation, the specification has demonstrated that, coupled with the information known in the art, one

of ordinary skill in the art would use the presently claimed compounds for inhibiting CDK2 *in vivo* without undue experimentation.

In addition, Applicants have concurrently filed a Declaration under 37 C.F.R. §1.132 ("Rule132 Declaration") with additional experimental data showing inhibitory effects of the inventive compounds on the proliferation of HeLa cells, which is a common model used in the art and correlates to the inhibitory effects of CDK2 *in vivo*. *See* Cancer Research 2006 article, pages 4302-4303 (Figure 2C) and 4305-4306.

As the Examiner has failed to point to any evidence rebutting the correlation between the inhibition of the kinase CDK2 *in vitro* and *in vivo,* and as the Examiner's analysis of Wands factors in support of the lack-of-enablement rejection on pages 4-9 of the Office Action is directed to the method of treating cancer, while claims 9-16 as currently amended recite a "method for inhibiting CDK2 *in vivo*", this rejection is improper. Accordingly, Applicants respectfully request this rejection of claims 9-16 be withdrawn.

B. Claims 17-25

The Examiner also rejects claims 17-25 under 35 U.S.C. § 112, first paragraph, for lack of enablement. Office Action, pages 4-9. Specifically, the Examiner alleges that, by analyzing each Wands factor, one of ordinary skill in the art would not be able to practice the presently claimed invention without undue experimentation. *Id.* Applicants respectfully disagree and traverse this rejection for at least the following reasons.

Here, the specification clearly indicates that "[i]t is known from literature that in the case of neoplastic diseases such as cancer, there is a connection between the therapy of said diseases and the inhibition of [the kinase] CDK2." Specification,

paragraph [004]. Further, Cancer Research 2006 article indicates, on page 4299, that "[t]he expression and catalytic activity of cyclin E/CDK2 complexes is elevated in multiple tumor types" and that "the majority of the published data suggest that inhibition of cyclin/CDK complex may prevent or delay tumor progression in cancer patients." Therefore, if a compound shows an inhibitory effect on the kinase CDK2, one of ordinary skill in the art would reasonably believe that it can be used for the treatment of cancer based on the information known in the art.

The Examiner has failed to point to any evidence rebutting the correlation between the treatment of cancer and the inhibition of the kinase CDK2.

In addition, because of the known correlation between the treatment of cancer and the inhibition of the kinase CDK2 Applicants respectfully disagree with the Examiner's analysis of the Wands factors for determining whether undue experimentation is necessary to practice the presently claimed invention as discussed below.

Applicants respectfully submit that the "quantity of experimentation necessary" is limited to the number of assays to be conducted to show the inhibition of the kinase CDK2. The specification in paragraphs [0301]-[0303] and the concurrently-filed Rule 132 Declaration provide detailed direction and guidance on how to perform the assays to show the inhibition of the kinase CDK2. Routine testing does not arise to the level of undue experimentation. *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988).

Further, "[t]he fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation." M.P.E.P.

§ 2164.01 (citation omitted). Applicants respectfully submit that the assays disclosed in paragraphs [0301]-[0303] of the specification and the concurrently-filed Rule 132 Declaration are routine experimentations known in the art.

The specification in paragraphs [0301]-[0303] and the concurrently-filed Rule 132 Declaration also disclose working examples, showing the inhibitory effects of the inventive compounds on the kinase CDK2.

Finally, contrary to the Examiner's allegation, the level of one of ordinary skill in the art is high. The Examiner alleges that "[t]he skill level in this art is extremely very low. To this day, there is no magic bullet that can treat cancer cells in general." Office Action, page 9. Applicants fail to understand how the lack of a magic bullet for cancer treatment has any bearing on the level of skill in the art. The M.P.E.P. clearly instructs that "[f]actors that may be considered in determining level of ordinary skill in the art include (1) the educational level of the inventor; (2) type of problems encountered in the art; (3) prior art solutions to those problems; (4) rapidity with which innovations are made; (5) sophistication of the technology; and (6) educational level of active workers in the field." M.P.E.P. § 2141.03.

Applicants respectfully submit that the educational level of the inventor is high, usually requiring a Ph.D. degree. The type of problems encountered in the art varies but the problems are difficult and the technology is sophisticated. Therefore, the level of ordinary skill in the art is high.

Accordingly, Applicants respectfully request the rejection of claims 17-25 be withdrawn.

V. Conclusion

In view of the foregoing amendments and remarks, Applicants respectfully request reconsideration of this application, and the timely allowance of the pending claims.

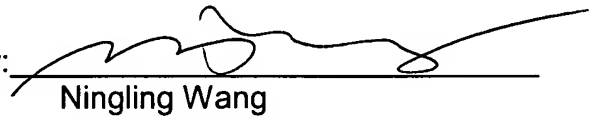
If the Examiner believes a telephone conference would be useful in resolving any outstanding issues, the Examiner is invited to call the Applicants' undersigned representative at (202) 408-4218.

If there is any fee due in connection with the filing of this response, please charge the fee to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: July 25, 2006

By: 

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Attachment:
Payton et al., "Discovery and Evaluation of Dual CDK1 and CDK2 Inhibitors," Cancer Research, 66(8), 2006, pages 4299-4308.

Discovery and Evaluation of Dual CDK1 and CDK2 Inhibitors

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Abstract

In eukaryotic cells, cyclin-dependent kinase (CDK) complexes regulate the temporal progression of cells through the cell cycle. Deregulation in the cell cycle is an essential component in the evolution of cancer. Here, we validate CDK1 and CDK2 as potential therapeutic targets using novel selective small-molecule inhibitors of cyclin B1/CDK1 and cyclin E2/CDK2 enzyme complexes (CDKi). Flow cytometry–based methods were developed to assess intracellular retinoblastoma (Rb) phosphorylation to show inhibition of the CDK pathway. Tumor cells treated with CDK inhibitors showed an overall decrease in cell proliferation, accumulation of cells in G_1 and G_2, and apoptosis in a cell line–specific manner. Although CDK inhibitors activate p53, the inhibitors were equipotent in arresting the cell cycle in isogenic breast and colon tumor cells lacking p53, suggesting the response is independent of p53. *In vivo*, the CDK inhibitors prevented the growth of colon and prostate tumors, blocked proliferation of tumor cells, and inhibited Rb phosphorylation. The discovery and evaluation of novel potent and selective CDK1 and CDK2 inhibitors will help delineate the role that CDK complexes play in regulating tumorigenesis. (Cancer Res 2006; 66(8): 4299-308)

Introduction

Deregulation of the cell cycle is one of the hallmarks of tumor formation and progression. Cyclins E1 and E2 bind and activate the catalytic activity of CDK2, and overexpression of cyclin E transforms immortalized cells in culture, induces chromosome instability, and stimulates tumor formation in transgenic mice (1–4). Recent reports have shown that elevated expression of cyclin E1 in primary tumors correlates with poor survival rates for breast cancer patients (5, 6). In addition, cyclin E2 is associated with a group of 70 genes whose expression in node-negative primary breast carcinomas correlates with poor prognosis. The expression of this gene set has outperformed all other clinical variables in predicting the likelihood of distant metastases within 5 years (7, 8). The expression and catalytic activity of cyclin E/CDK2 complexes is elevated in multiple tumor types, including primary breast tumors (9–12). Cyclin B1/CDK1 complexes have been implicated in a number of cell cycle processes, including the monitoring of both the DNA structure checkpoints during late G_2 and the spindle assembly checkpoint during mitosis (13, 14). Aberrant expression of CDK1 and cyclin B1 has been described for a number of primary tumors and, in some cases, seems to correlate with patient survival rates (15–17).

Recent reports have shown that ablation of CDK2 expression by RNA interference (RNAi), antisense, and genetic knockout does not alter cell cycle progression, and, surprisingly, the CDK2 homozygous null mice are viable with normal life spans (18–21). Although CDK2 knockout data show that CDK2 may not be required for cell proliferation, a recent report suggests that melanocytes may be uniquely dependent on CDK2 for proliferation (19). Retroviral-mediated RNAi suppression of CDK2 in melanoma tumor-derived cell lines inhibits cell cycle progression, proliferation, and colony formation, strongly suggesting that CDK2 may be an essential cell cycle regulator in melanomas (18, 19). Although CDK1 activity is clearly required for G_2 and mitosis in lower organisms and tumor-derived cell lines, the effect of deleting CDK1 in transgenic mice has not yet been reported. CDK1 is the only mammalian CDK complex present and active during late G_2 and mitosis; therefore, the loss of CDK1 would likely result in embryonic lethality.

CDK complexes regulate cell cycle progression by phosphorylating downstream substrates required for G_1-S and G_2-M transit. One of the substrates for G_1 cyclin/CDK complexes is the retinoblastoma protein (Rb), a tumor suppressor implicated in negatively regulating E2F-mediated transcriptional responses (22–24). Although it is not clear if individual CDK complexes recognize specific phosphorylation sites on Rb, it is clear that CDK-mediated Rb phosphorylation is a critical step in regulating E2F transcriptional responses. Cyclin B1/CDK1 kinase complexes regulate late G_2 and mitosis by phosphorylating substrates and triggering dramatic structural reorganization of the nuclear envelope, spindle apparatus, and actin cytoskeleton and induction of chromosomal DNA condensation (25). One of the substrates for CDK1 complexes is PP1-α phosphatase, which is phosphorylated at Thr^{320} by cyclin B1/CDK1 during metaphase. Phosphorylation on Thr^{320} is believed to inhibit PP1-α phosphatase activity and thereby contribute to the increased phosphorylation of proteins critical in orchestrating mitotic progression (26).

To date, the majority of the published data suggest that inhibition of cyclin/CDK complexes may prevent or delay tumor progression in cancer patients. There are currently a number of ongoing clinical trials using CDK inhibitors, including Flavopiridol, UCN-01, CYC202 (racemic Roscovitine), and BMS-387032 (27). The compounds have a range of potencies against recombinant CDK enzyme complexes and tumor cells in *in vitro* and *in vivo* assays

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-05-2507

(28, 29). However, many of these small-molecule CDK inhibitors either lack potency, selectivity, or suitable physical properties. For example, a small-molecule CDK inhibitor (BMS-387032) currently in clinical trials has IC_{50}s of 480, 48, and 925 nmol/L against recombinant cyclin B1/CDK1, cyclin E/CDK2, and cyclin D/CDK4 kinase complexes, respectively (30). In addition, the maximum tolerated dose (MTD) of BMS-387032 is 48 mg/kg, and efficacy is observed in an A2780 tumor xenograft model at 36 to 48 dosed once a day for 8 days, showing a narrow therapeutic index (31). Another small-molecule CDK inhibitor currently in clinical trials, racemic Roscovitine, has an IC_{50} of 0.65 μmol/L against CDK1, an average IC_{50} of 0.7 μmol/L against CDK2 complexes, and an average IC_{50} of 16 μmol/L in growth inhibition assays against a panel of 60 tumor-derived cell lines from the National Cancer Institute (31). The R-isomer of Roscovitine, CYC202, has slightly greater potency against CDK2 complexes with an average IC_{50} of 0.4 μmol/L, is less potent against CDK1 complexes with an IC_{50} of 2.7 μmol/L, and has an average IC_{50} of 15.2 μmol/L in tumor cell growth inhibition assays (32, 33). *In vivo* tumor experiments showed CYC202 dosed thrice a day i.p. at 100 mg/kg for 5 days resulted in a 45% reduction in tumor volume at day 27 in a human colorectal LoVo tumor model (32). Therefore, there is still a need for potent and selective CDK inhibitors to test the idea that CDK complexes are required for the proliferation and survival of primary tumors. In this report, we describe the discovery and characterization of novel potent small-molecule inhibitors of cyclin E/CDK2 and cyclin B1/CDK1 complexes and discuss the possible therapeutic implications for CDK inhibition.

Materials and Methods

Biochemical assays. A primary biochemical screen was completed with recombinant cyclin E2/CDK2 (in house, baculovirus) using homogenous time-resolved fluorescence technology platform. The kinase reaction [10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L $MgCl_2$, 2 mmol/L glycerophosphate, 1 mmol/L DTT, 5 μmol/L ATP, 1 μmol/L biotinylated histone H1, 1 mmol/L EGTA, and 0.2 mg/mL bovine serum albumin (BSA) followed by the addition of 2 nmol/L cyclin E2/CDK2] was incubated for 60 minutes at room temperature. Detection of substrate phosphorylation was determined using streptavidin allophycocyanin (Prozyme, Sanleadro, CA) and europium anti-phosphothreonine proline antibody (Perkin-Elmer, Boston, MA). Compounds potent in the primary screen were tested using recombinant cyclin D3/CDK6 (Upstate Cell Signaling, Waltham, MA), cyclin B1/CDK1 (Biomol., Plymouth Meeting, PA), p25/CDK5 (in house, baculovirus), and GSK-3B (in house, baculovirus). Kinase activities were determined using substrates histone H1 and/or COOH-terminal Rb protein fragment (Upstate Cell Signaling). The kinase reactions [20 mmol/L Tris-HCl (pH 7.5), 12.5 mmol/L $MgCl_2$, 100 mmol/L NaCl, 1 mmol/L DTT, 125 μg/mL BSA, 2-3× K_m value ATP, 2-3× K_m value substrate, and 5 uCi [γ-^{33}P]ATP (Amersham Piscataway, NJ) followed by addition of 5 nmol/L recombinant enzyme] were incubated for 10 minutes at room temperature. The compounds were serially diluted over the appropriate concentration range and added to the kinase buffer before the addition of recombinant enzyme. The data were expressed as percent inhibition = 100 − [(signal with inhibitor − background) / (signal with DMSO vehicle − background)] × 100.

Human cells and cell culture. The tumor cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA). Human primary bone marrow mononuclear cells were obtained from Cambrex BioScience (Walkersville, MD). Cells were maintained at 37°C in an atmosphere of 5.0% CO_2.

CDK1 and CDK2 RNAi and kinase assay. RNAi triggers were synthesized for CDK1, CDK2, and single mismatch controls for both CDK1 and CDK2. RNAi triggers were transfected into cells using the liposome LipofectAMINE 2000 (Invitrogen). After 16 hours of incubation, the transfection medium was removed and replaced with fresh complete medium. The transduced U2OS cells were harvested on the third day after a 1-hour pulse with bromodeoxyuridine (BrdUrd; Invitrogen). Immunoprecipitations, kinase reactions, and Westerns were done as previously described (12). The control for the kinase assay was U20S cells treated with 1 μmol/L CDKi 277 for 24 hours.

Human p53 knockout cell lines. Plasmid vectors harboring small interfering RNA (siRNA) triggers for either human p53 or scrambled sequence control were transfected into MCF-7 cells and HCT116 using the liposome Fugene 6 (Roche, Indianapolis, IN). The cells were clonally selected in complete media supplemented with 400 μg/mL of G418 (Sigma, St. Louis, MO). Individual clones were then assessed for p53 protein levels.

Mitotic-arrested HeLa cell extract preparation. HeLa cells were treated with 0.1 μg/mL Nocodazole (Sigma) for 12 hours; the semi-adherent, mitotic cells were collected and removed by pipetting. Cells were then treated with inhibitor compounds at indicated concentrations for 1 hour and harvested for Western analysis as previously described (12).

Antibodies and Western blotting. Mouse monoclonal antibodies anti-total Rb, anti-underphospho-Rb, and anti-poly(ADP-ribose)polymerase (PARP; Asp^{214}) were obtained from BD Biosciences (San Jose, CA). Rabbit polyclonal antibodies anti-phospho-Rb (Ser^{807}/Ser^{811}), anti-total PP1-α, anti-phospho-PP1-α (Thr^{320}), anti-total p53, and anti-phospho-p53 (Ser^{15}) were obtained from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal antibodies anti-CDK2, anti-CDK1, and mouse monoclonal anti-p21 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody anti-actin (Sigma) was used as a protein loading control. *In vitro* cell line extracts were prepared for Western analysis using a modified radioimmunoprecipitation assay buffer (12).

Flow cytometry. The cell lines were treated with CDKi 277 [0.04-2.5 μmol/L (hypophosphorylated Rb, hypo-Rb), 0.0019-5 μmol/L (BrdUrd), and 0.5 μmol/L (time course)] or Roscovitine (Calbiochem EMD Biosciences, Inc., San Diego, CA; 0.41-100 μmol/L BrdUrd) for 24 hours of continuous exposure or harvested at 4, 8, 12, and 24 hours. The cells were pulsed with BrdUrd for 1 hour before cell harvest when appropriate. The cells were fixed, permeabilized, acid treated (only BrdUrd), and neutralized in preparation for intracellular staining. The cells were stained with antibodies for 2 hours using anti-BrdUrd-FITC (BD Biosciences) or anti-BrdUrd-alexa 647 (Invitrogen), anti-cyclin B1-FITC (BD Biosciences), active caspase 3-FITC (BD Biosciences), and anti-histone H3 p-Ser^{10}-Alexa 488 (U.S. Biological, Swampscott, MA) followed by DNA counterstaining with propidium iodide. The 96-well hypo-Rb assay was developed using the Colo205 cells. The cells were treated with inhibitor compounds (12-pt. dose range, 0.0025-50 μmol/L) for 5 hours. The cells were fixed and permeabilized in preparation for intracellular staining. The fixed cells were stained with anti-hypo-Rb-alexa 488 antibody for 2 hours at room temperature and counterstained with 7-AAD (BD Biosciences). Data was acquired using a 96-well plate AMS auto-sipper (Cytek, San Jose, CA) with FACScan flow cytometer (BD Biosciences).

Tumor xenografts. All *in vivo* experiments were conducted in accordance with institutional animal care and use committee. Eight- to 10-week-old female CD1 nude mice (Charles Rivers Laboratories, Wilmington, MA) were used in all studies. Mice were injected s.c. with 2×10^6 tumor cells. Tumor volume was calculated as length × $(\text{width})^2$ and expressed in mm^3. Mice were euthanized with CO_2 asphyxiation. Results are described as mean ± SE. The data were statistically analyzed with factorial ANOVA followed by Scheffe's post hoc analysis for repeated measurements (StatView v5.0.1, SAS Institute, Cary, NC). The mice for the short-term tumor xenografts were treated i.p. with CDKi 277 at 50 mg/kg once a day (QD) and vehicle QD for 4 days, or Taxotere at 20 mg/kg QD for 2 days. Tumors were harvested on day 5, 2 hours before the harvest mice were injected i.v. through the tail vein with 0.2 mL of BrdUrd at 2 mg/mL (Invitrogen). Tumors were excised, minced, and processed in preparation for flow cytometry and Western analysis (12).

Results

Inhibition of CDK Expression Reduces Tumor Cell Proliferation

To determine the cellular effect of selectively inhibiting CDK complexes, RNAi-based suppression methods were used to specifically target CDK1 or CDK2. As shown in Fig. 1, RNAi-mediated inhibition of CDK2 expression and kinase activity modestly increased the percentage of cells in G_2-M and had little or no effect on BrdUrd incorporation or the overall cell cycle profile of asynchronously dividing U2OS osteosarcoma cells. These data are consistent with previous results targeting CDK2 with antisense oligonucleotides in U2OS cells (18). In contrast, inhibition of CDK1 expression decreased the percentage of U2OS cells in the S phase by 26% and increased the percentage of cells in G_2-M by almost 3-fold when compared with mismatch-treated controls. Inhibition of both CDK2 and CDK1 expression produced results similar to U2OS cells treated with CDK1 RNAi alone (Fig. 1*C*).

Antisense-based suppression of CDK2 and CDK1 in breast tumor–derived MDA-MB-453 cells showed that inhibition of CDK2 expression led to a 29% decrease in BrdUrd-positive, S-phase cells relative to the mismatch antisense control (data not shown). Suppression of CDK1 expression in MDA-MB-453 cells resulted in a 48% decrease in BrdUrd-positive, S-phase cells, a 47% decrease in G_2-M, and a 164% increase in the sub-G_1 DNA content relative to the mismatch antisense control (data not shown). The decrease in S and G_2-M phases with CDK1 antisense was likely due to an increase in cell death. Overall, these results show that selective inhibition of CDK2 expression had a very modest effect on cell proliferation, whereas suppression of CDK1 expression decreased the percentage of cells in the S phase for both U2OS and MDA-MB-453 cells. Inhibition of CDK1 expression also led to either cell death in MDA-MB-453 cells or an increase in the percentage of U2OS cells in the G_2-M phase.

Discovery of Small-molecule CDK Inhibitors (CDKi)

Suppression of CDK1 using RNAi suggests an essential role in cell cycle progression; CDK2 suppression alone could not inhibit cell cycle progression, suggesting the possibility of CDK pathway redundancy. However, previous reports clearly showed that both dominant-negative mutant CDK2 and CDK2 inhibitory peptides blocked tumor cell proliferation (34, 35). Therefore, we designed a high-throughput screen for dual CDK2 and CDK1 inhibitors. To discover novel small-molecule inhibitors for CDK2 a high-throughput screen was completed using active recombinant cyclin E2/CDK2 complexes. As shown in Table 1A and B, a representative thiazole urea [(*N*_(2_(4_pyridinyl)_1,3_thiazol_4_yl)_*N'*_(6_(((2*R*)_2_pyrrolidinylmethyl)oxy)_2_pyridinyl)urea)] small-molecule compound, CDKi 277, potently inhibited CDK2, CDK1, and CDK5 kinase activity with IC_{50}s below 10 nmol/L. CDKi 277 is 10-fold more selective for CDK2 than CDK6 or GSK-3β. Inhibition of GSK-3β enzyme with CDKi 277 is consistent with the fact the GSK-3 kinases, and CDKs have structural similarities in the

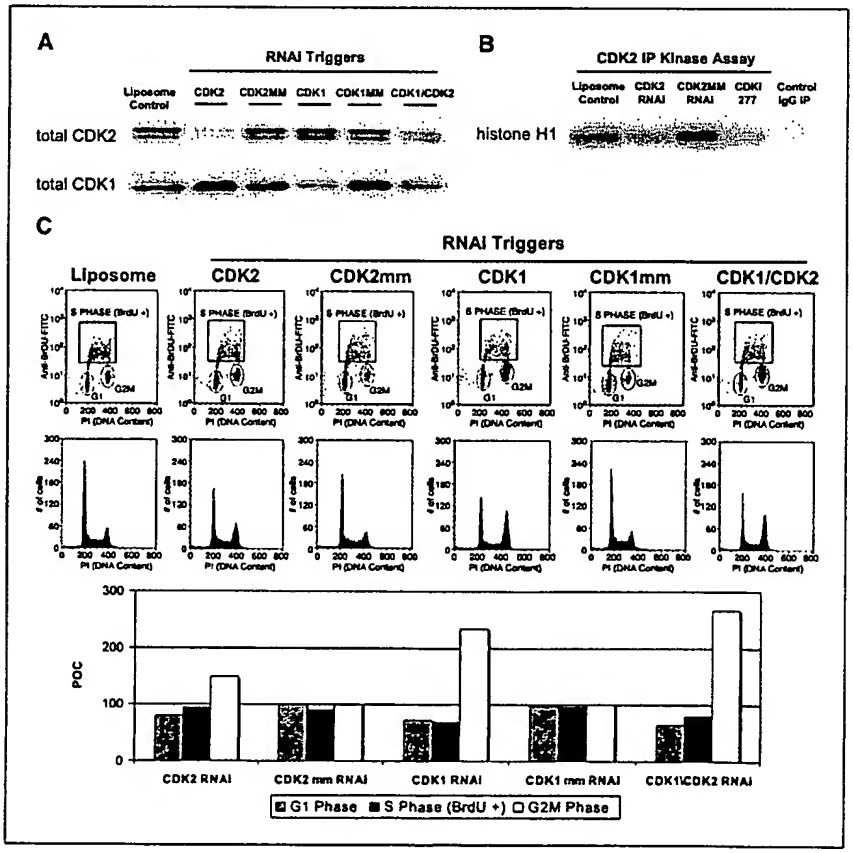
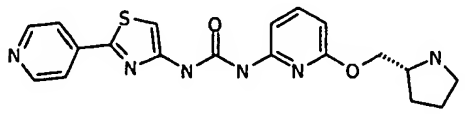


Figure 1. RNAi-mediated suppression of CDK1 and CDK2 in U2OS osteosarcoma tumor cells. *A*, Western analysis of CDK1, CDK2, CDK1/2, and CDK mismatch (*MM*) controls. *B*, immunoprecipitated CDK2 kinase activity was measured by using histone H1 substrate. Rabbit isotype antibody and in cell treatment with CDKi 277 were used as controls. *C*, BrdUrd-coupled cell cycle analysis of G_1, S (*BrdU+*), and G_2-M phases are depicted in the scatter plots [BrdUrd versus propidium iodide (*PI*)] and DNA histograms (*PI*) for CDK1, CDK2, CDK1/2, CDK mismatch, and liposome control (same transfection as in *A*). Cell cycle phases were represented as percentage of control (liposome alone).

Table 1.

A. Structure of CDKi 277, a representative thiazole urea small-molecule CDK inhibitor



B. Biochemical enzyme IC_{50}s and kinase selectivity

Biochemical assay	IC_{50} (μmol/L)	Fold selectivity
CDK2-E2	0.004	1
CDK1-B1	0.008	2
CDK5-p25	0.005	1.3
CDK6-D3	0.051	12.8
GSK-3β	0.041	10.3
9 Serine/threonine kinases	>5.0	>500
5 Tyrosine kinases	>5.0	>500
10 Serine/threonine kinases	>50 POC at 10 μmol/L	
17 Tyrosine kinases	>50 POC at 10 μmol/L	

C. Inhibition of Rb, PP1α phosphorylation, and cell proliferation

In cell phosphorylation assays
Rb phosphorylation cell assay IC_{50} = 0.163 μmol/L
PP1α phosphorylation cell assay IC_{50} = 0.305 μmol/L
In vitro cell proliferation assays
Mean BrdUrd IC_{50} = 0.234 μmol/L (cell lines tested, n = 21)

ATP binding domains. To determine the selectivity of CDKi 277 against additional kinases, we screened a panel of 41 serine/threonine and tyrosine kinases and showed that CDKi 277 is selective against these additional kinases (Table 1B). The thiazole urea structural series of compounds are competitive with respect to ATP, noncompetitive with respect to substrate and are reversible (data not shown).

Inhibitors Block Phosphorylation of CDK Substrates in Tumor Cells

pRb phosphorylation. CDK inhibitors having IC_{50}s <10 nmol/L in the CDK1 and CDK2 kinase assays were tested for their ability to inhibit CDK activity in cells using a novel 96-well plate flow cytometry–based Rb phosphorylation assay. Rb is a proximal substrate for G_1 CDK complexes and the phosphorylation state of Rb correlates with its ability to function as a tumor suppressor. A monoclonal antibody recognizing the hypophosphorylated or underphosphorylated form of Rb was chosen to develop the phospho-Rb flow cytometry assay. CDK inhibitors shift Rb to a hypophosphorylated (hypo-Rb) state where it is recognized by the hypo-Rb–specific monoclonal antibody. The whole cell flow cytometry approach using threshold gating provided superior sensitivity and reproducibility, compared with cell extract–based methods. A panel of cell lines were evaluated for expression of total Rb protein; the colon tumor-derived cell line Colo205 proved to be the best candidate based on Rb protein levels (Fig. 2*A*).

Colo205 cells treated with CDKi 277 show a clear dose-dependent increase in hypo-Rb (*red*, *y axis*) using the phospho-Rb flow cytometry assay (Fig. 2*B*). The cellular phospho-Rb IC_{50} for CDKi 277 was 0.163 μmol/L (Fig. 2*B*). Time course experiments have shown that Rb shifted to a hypophosphorylated state within 30 minutes of compound treatment (data not shown). To reduce the likelihood that inhibition of Rb phosphorylation was due to a cell cycle arrest, cells were treated with compound for 5 hours, which is before any substantial CDK inhibitor, mediated changes in cell proliferation (Fig. 3*B*). Western blot analysis with phospho-Rb (Ser^{807} and Ser^{811}) and hypo-Rb antibodies confirmed the flow cytometry–based IC_{50} for CDKi 277 (Fig. 2*B*).

PP-1α phosphatase phosphorylation. PP-1α phosphatase is a direct substrate for cyclin B1/CDK1 complexes; the CDK inhibitor olomoucine has been shown to block the phosphorylation of PP-1α on Thr^{320} in mitotic cell extracts (26). To determine if CDKi 277 inhibited intracellular CDK1 activity, HeLa cells were synchronized in mitosis with nocodazole, and the semiadherent mitotic cells were harvested and treated with CDKi 277 for 1 hour. As shown in Fig. 2*C*, proliferating HeLa cells show

very little phosphorylation of PP-1α, whereas the nocodazole-treated cells show a dramatic increase in PP-1α phosphorylation. Figure 2C shows that CDKi 277 clearly triggered a dramatic dose-dependent decrease in phosphorylation of PP-1α in cells released from a mitotic nocodazole block. At doses of ≥0.625 μmol/L, the phosphorylation on Thr^{320} was completely inhibited with no change in the total amount of PP-1α. Densitometry measurements of signal intensity for the bands depicting phosphorylation of PP-1α on Thr^{320} indicate an IC_{50} of 0.3 μmol/L for CDKi 277 (Fig. 2C).

Our results establish the use of screening CDK inhibitors using endogenous cellular substrates in their native forms. The phospho-Rb flow cytometry assay identified small molecules capable of inhibiting CDK-mediated phosphorylation of intracellular Rb. In addition, phosphorylation of PP-1α on Thr^{320} has proven to be an excellent secondary assay to show inhibition of CDK1 activity in a tumor-derived cell line.

CDK Inhibitors Block Cells in G_1 and G_2 and Induce Apoptosis

To determine the effect of CDK inhibition on cell proliferation, we treated a panel of tumor cell lines and normal nontransformed cells with CDKi 277 and Roscovitine and examined their cell cycle profiles after 24 hours of treatment. CDKi 277 and Roscovitine inhibited BrdUrd uptake in all of the cell lines. For CDKi 277 the IC_{50}s ranged from 0.09 to 0.7 μmol/L with a mean IC_{50} of 0.243 μmol/L, whereas Roscovitine IC_{50}s ranged from 12 to 35 μmol/L with a mean IC_{50} of 19.7 μmol/L. On average, the CDKi 277 inhibitor was ~77-fold more potent compared with Roscovitine (Table 2; Fig. 3A). The 24-hour cellular phenotypes were classified as either cytostatic (G_1 and G_2-M arrest) or cytotoxic (>20% cell death measured by % sub-G_1 DNA content). Although the CDK inhibitors differ dramatically in terms of potency, both compounds triggered similar cell cycle responses in both tumor

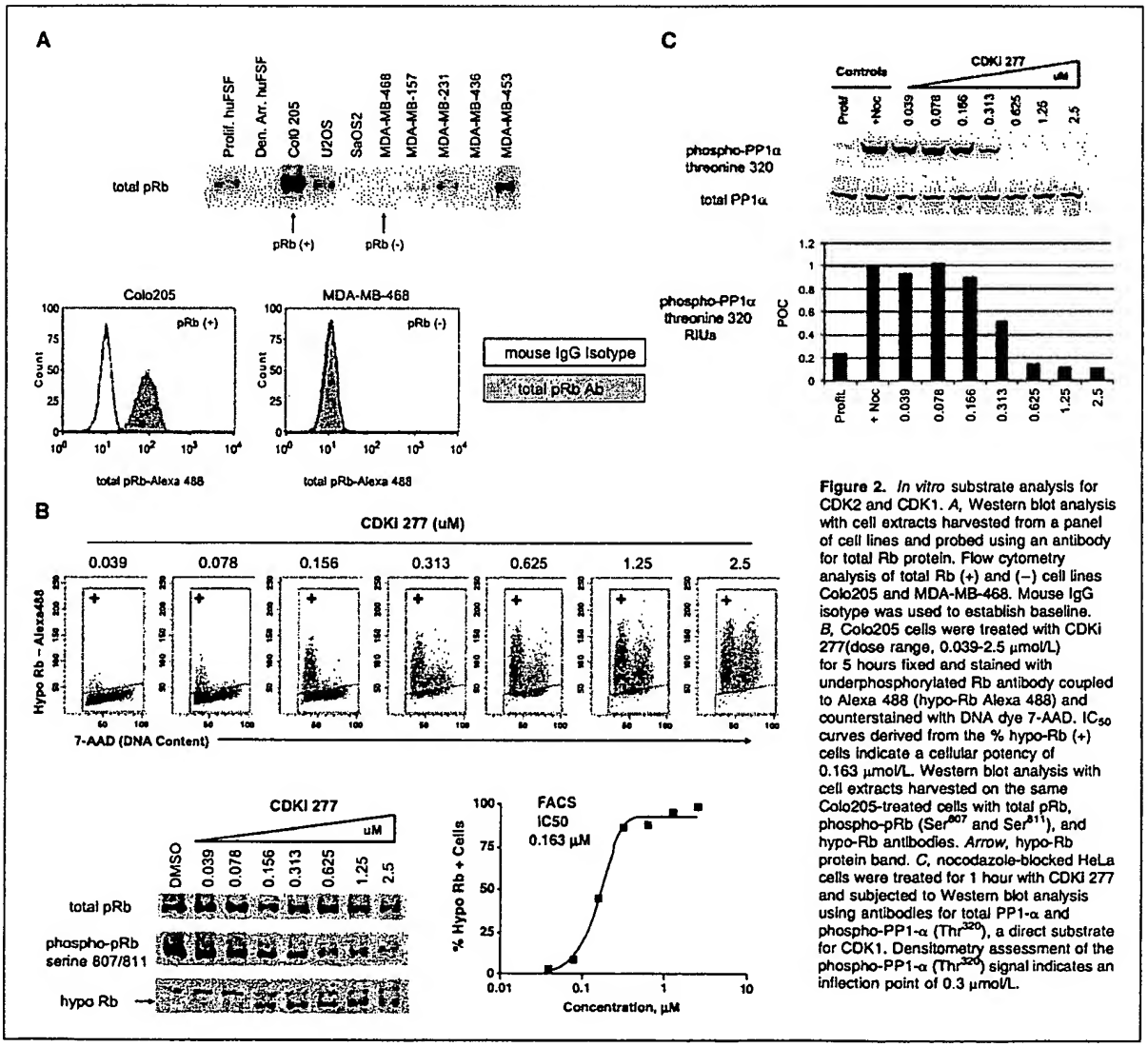


Figure 2. *In vitro* substrate analysis for CDK2 and CDK1. *A*, Western blot analysis with cell extracts harvested from a panel of cell lines and probed using an antibody for total Rb protein. Flow cytometry analysis of total Rb (+) and (−) cell lines Colo205 and MDA-MB-468. Mouse IgG isotype was used to establish baseline. *B*, Colo205 cells were treated with CDKi 277(dose range, 0.039-2.5 μmol/L) for 5 hours fixed and stained with underphosphorylated Rb antibody coupled to Alexa 488 (hypo-Rb Alexa 488) and counterstained with DNA dye 7-AAD. IC_{50} curves derived from the % hypo-Rb (+) cells indicate a cellular potency of 0.163 μmol/L. Western blot analysis with cell extracts harvested on the same Colo205-treated cells with total pRb, phospho-pRb (Ser^{807} and Ser^{811}), and hypo-Rb antibodies. *Arrow*, hypo-Rb protein band. *C*, nocodazole-blocked HeLa cells were treated for 1 hour with CDKi 277 and subjected to Western blot analysis using antibodies for total PP1-α and phospho-PP1-α (Thr^{320}), a direct substrate for CDK1. Densitometry assessment of the phospho-PP1-α (Thr^{320}) signal indicates an inflection point of 0.3 μmol/L.

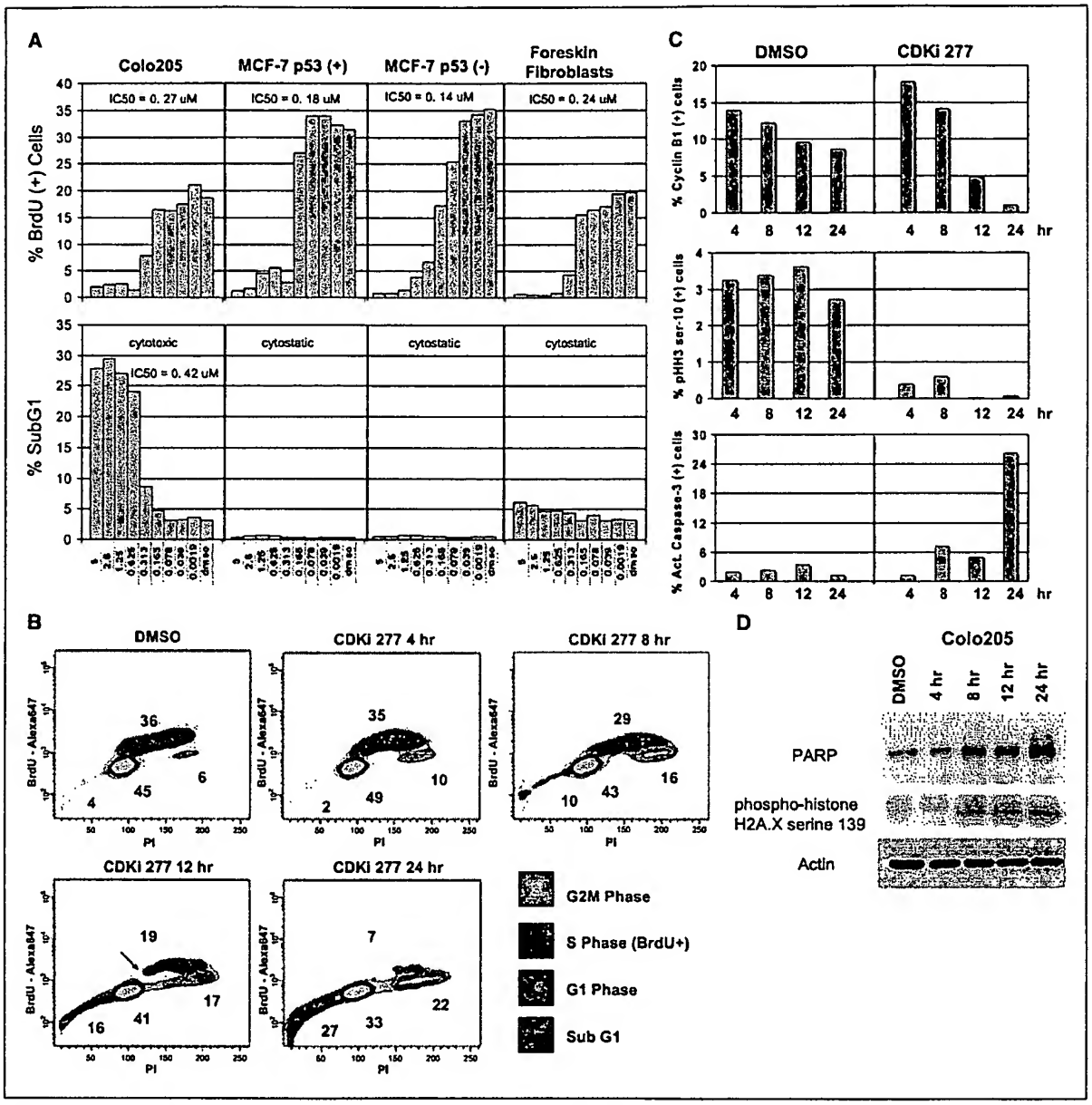


Figure 3. CDK inhibitors block cells in G_1 and G_2 and induce apoptosis. *A*, 24-hour full dose-response using CDKi 277 was done on tumor cell lines Colo205, MCF-7 p53 (+), MCF-7 p53 (−), and normal nontransformed foreskin fibroblasts. Flow cytometry BrdUrd-coupled DNA staining was used to simultaneously measure cell proliferation and cell death (sub-G_1 DNA content). *B*, time course of Colo205 cells treated with CDKi 277 at 0.5 μmol/L. Cell cycle stage and sub-G_1 were determined (boldface number represents % cells/phase or sub-G_1). For the 12-hour sample, the arrow (→) indicates complete inhibition of G_1 cells traversing into the S phase (*BrdU+*). *C*, time course of Colo205 cells treated with either 0.5 μmol/L CDKi 277 or DMSO. Flow cytometry analysis of the same Colo205-treated cells using DNA-coupled staining with cyclin B1, phospho-histone H3 (Ser^{10}) and active caspase-3 antibodies. *D*, Western blot analysis was done on protein extracts using the following cleaved-PARP, histone H2AX (Ser^{139}), and actin. Actin antibody was used as a protein loading control.

cell lines and in normal bone marrow–derived leukocytes, the one exception was the uterine tumor-derived cell line MES-SA (Table 2). Interestingly, osteosarcoma cell lines SaSO2 (Rb^-) and U2OS (Rb^+) treated with CDKi 277 had comparable IC_{50}s (~0.3 μmol/L) and cellular phenotypes (cytotoxic), indicating that CDKi 277 inhibits cell cycle progression irrespective of Rb status. Additionally, MCF-7 and HCT116 $p53^{+/-}$ isogenic tumor cell lines treated with CDKi 277 exhibited nearly identical cell cycle responses, suggesting that p53 may not play an essential role in regulating the cell arrest phenotype in G_1 or G_2 (Table 2; Fig. 3*A*).

To determine the kinetics of cell cycle inhibition, Colo205 cells were treated with CDKi 277 over a 24-hour time period, harvested at various time points, and evaluated using the following end points; cell cycle profiles, BrdUrd analysis of S phase, apoptosis markers, mitotic markers, and p53 pathway activation. Figure 3*B* shows that Colo205 cells treated with CDKi 277 show a time-dependent decrease in % S phase ($BrdUrd^+$) and an increase in % G_2-M and % sub-G_1. G_1 cells decreased only modestly; however, by 12 hours, G_1 cells were completely blocked from entering into the S phase. To determine the cause of the precipitous drop in proliferation upon treatment with CDKi 277, Colo205 cells were assessed for the following apoptosis end points: cleaved caspase-3, cleaved PARP, and phosphorylation of histone H2A.X on Ser^{139} (Fig. 3*D*). The phosphorylation of histone H2AX on Ser^{139} by ATM occurs in response to double-strand DNA breaks and apoptosis-mediated genome fragmentation (36, 37). Colo205 cells treated with CDKi 277 show a time-dependent increase in all three apoptotic indicators (Fig. 3*B* and *D*).

To gain a clearer understanding of whether cells were arrested in G_2 or mitosis, tumor cells were exposed to CDKi 277 and analyzed by flow cytometry using antibodies against cyclin B1, phosphorylated histone H3, and MPM-2. Figure 3*C* shows a dramatic decrease in cyclin B1 and phospho-histone H3 staining, indicating that the cell cycle arrest occurred in G_2 before entering into mitosis. The decrease in phospho-histone H3 staining is likely caused by inhibition of downstream CDK1 effectors. As an additional measure, cells treated with compound were stained for tubulin and DNA and analyzed by fluorescent microscopy. We did not observe condensed chromosomes or metaphase plate formation in compound-treated cells, which is consistent with cells arrested in G_2 and not mitosis (data not shown).

Cell Cycle Arrest Mediated by CDK Inhibitors Is p53 Independent

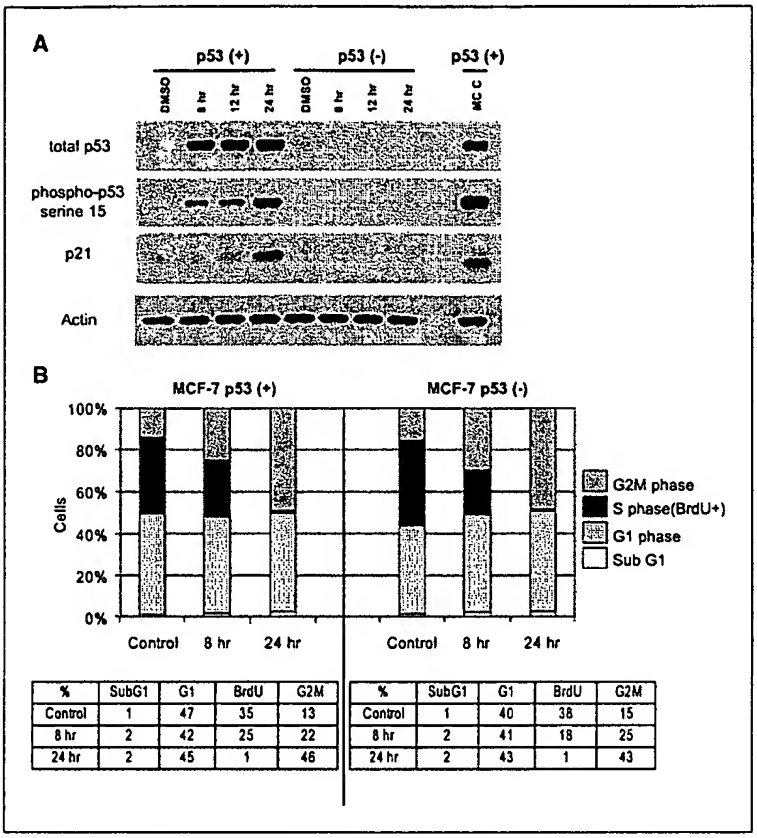
To determine if the antiproliferative effects of CDKi 277 were dependent upon activation of p53, isogenic MCF-7 breast tumor cells lacking p53 were generated using a stable transducer vector harboring a siRNA targeting p53 gene expression. As a control, scrambled siRNA was stably transfected into the parental MCF-7 cells. MCF-7 p53-positive cells treated with CDKi 277 showed a time-dependent accumulation of both total p53 protein and phosphorylated p53 protein on Ser^{15} (Fig. 4*A*). Ser^{15} is a major site of p53 phosphorylation in response to DNA damage, and ATM/ATR kinases have been shown to phosphorylate p53 on Ser^{15} in response to CDK2 inhibition (38, 39). As expected, treatment of MCF-7 p53-negative cells with CDKi 277 did not increase the expression of p53 or p21 protein (Fig. 4*A*). In the MCF-7 p53-positive cells, p21 induction was visible only after 24 hours of compound treatment, clearly showing the decrease in BrdUrd incorporation resulting from CDK inhibition preceded the increase in p21 protein (Fig. 4*A* and *B*). Interestingly, the flow cytometry analysis showed similar G_1 and G_2 cell cycle arrest profiles for both MCF-7 p53-positive and p53-negative cells (Fig. 4*B*), indicating that although CDKi 277 activates the p53 pathway, the activation of p53 is not required for CDKi 277 to inhibit the cell cycle.

Inhibition of CDK Activity Suppresses the Growth of Human Tumors *In vivo*

Short-term human tumor cell *in vivo* proliferation assay. CDK inhibitors exhibiting potent inhibitory activity in the cell-based proliferation and substrate phosphorylation assays and having acceptable pharmacokinetic and physical properties were tested in a short-term *in vivo* tumor cell proliferation assay. Colo205

Table 2. Twenty-four-hour BrdUrd IC_{50}s (μmol/L) and cellular phenotypes for CDKi 277– and Roscovitine-treated cell lines

Cell lines (origin)	CDKi 277		Roscovitine	
	IC_{50} (μmol/L)	Cellular phenotype	IC_{50} (μmol/L)	Cellular phenotype
Daudi (B lymphoblast, Burkitt's lymphoma)	0.171	Cytostatic	19.2	Cytostatic
HL60 (acute promyelocytic leukemia)	0.129	Cytotoxic	20.5	Cytotoxic
K562 (chronic myelogenous leukemia)	0.438	Cytotoxic	35	Cytotoxic
Jurkat (acute T-cell leukemia)	0.14	Cytotoxic	27.8	Cytotoxic
Normal bone marrow (leukocytes, $CD45^+$)	0.09	Cytotoxic	15.9	Cytotoxic
HS294 (melanoma)	0.221	Cytotoxic	16.6	Cytotoxic
A375 (melanoma)	0.12	Cytotoxic	19.9	Cytotoxic
MiaPaca2 (pancreatic)	0.116	Cytostatic	12.6	Cytostatic
PC-3 (prostate)	0.251	Cytostatic	12	Cytostatic
SaSO2 (osteosarcoma) −Rb	0.32	Cytotoxic	17.1	Cytotoxic
U2OS (osteosarcoma) +Rb	0.28	Cytotoxic	28.3	Cytotoxic
Colo205 (colon)	0.27	Cytotoxic	18.6	Cytotoxic
Colo320 (colon)	0.241	Cytotoxic	15.9	Cytotoxic
HCT116 $p53^+$ (colon)	0.29	Cytostatic	13.7	Cytostatic
HCT116 $p53^-$ (colon)	0.31	Cytostatic	23.7	Cytostatic
MCF-7 $p53^+$ (breast)	0.18	Cytostatic	Not done	
MCF-7 $p53^-$ (breast)	0.14	Cytostatic	Not done	
MDA-MB-231 (breast)	0.137	Cytostatic	20.8	Cytostatic
MES-SA (uterine)	0.31	Cytostatic	25	Cytotoxic
MES-SA/Dx5 (uterine, MDR^+)	0.7	Cytotoxic	12.5	Cytotoxic
Normal foreskin fibroblast (fibroblast)	0.24	Cytostatic	Not done	
Mean BrdUrd IC_{50} (μmol/L)	0.243		19.73	



%	SubG1	G1	BrdU	G2M
Control	1	47	35	13
8 hr	2	42	25	22
24 hr	2	45	1	46

%	SubG1	G1	BrdU	G2M
Control	1	40	38	15
8 hr	2	41	18	25
24 hr	2	43	1	43

Figure 4. The cell cycle arrest mediated by CDK inhibitors is p53 independent. Time course of pseudoisogenic MCF-7 p53 (+) and p53 (−) cells treated with CDKi 277at 0.5 μmol/L. *A*, Western blot analysis was done on protein extracts using the following antibodies total p53, phospho-p53 (Ser^{15}), and p21. Actin antibody was used as a protein loading control. As a positive control, MCF-7 p53 (+) cells were treated with 8 μg/mL mitomycin C (*MCC*). *B*, flow cytometry analysis of the same MCF-7 p53 (+) and p53 (−) cells treated with CDKI 277 using BrdUrd-coupled DNA staining.

tumor-bearing CD1 nude mice were treated with CDKi 277 for 4 days. Tumor samples were harvested and processed for flow cytometry–based BrdUrd analysis. The mice treated with either CDKi 277 at 50 mg/kg QD or taxotere at 20 mg/kg QD showed a significant decrease in Colo205 tumor cell proliferation when compared with the vehicle control-treated mice (see Supplemental Fig. S1).

CDKi 277 is active in long-term human tumor xenograft models. Given that CDKi 277 potently inhibited Colo205 tumor cell proliferation in the short-term *in vivo* assay, it was considered an appropriate candidate for long-term tumor xenograft models. Figure 5*A* shows that CDKi 277 reduced the rate of Colo205 tumor growth when dosed twice a day (BID) at 12.5 and 25 mg/kg for three cycles of 4 days on and 1 day off. Tumor volumes for CDKi 277–treated mice (n = 10 per dose group) showed stasis at 12.5 mg/kg and regression at 25 mg/kg when compared with the vehicle control group. The mice seemed to tolerate the 12.5 mg/kg dose with 100% survival and no significant weight loss; but at 25 mg/kg, survival rates dropped dramatically after the start of the third cycle of treatment. A follow-up study using the Colo205 tumor model was completed using a less frequent dosing schedule; CDKi 277 was dosed BID at 17.5 and 12.5 mg/kg for 3 days per week for 3 weeks. CDKi 277 induced complete tumor stasis over the course of treatment with no adverse effects on body weights or survival (data not shown). In addition, Fig. 5*B* shows in an established PC-3 prostate tumor xenograft model that treatment with CDKi 277 dosed at 12.5 mg/kg BID for 2 days on and 4 days off for five cycles inhibited tumor growth. In a separate arm of the study, we observed tumor regressions in mice treated with Taxotere. Both cell cycle inhibitors were well tolerated over the course of the study. Studies we conducted with CDK inhibitor Roscovitine showed 35% tumor growth inhibition in an established PC-3 tumor model (data not shown). Roscovitine was given as a continuous s.c. infusion using osmotic minipumps at 10 mg/mL (2 mg/wk) for 3 weeks. Osmotic minipumps were chosen because of the demanding dose and schedule requirements for i.p. administration and formulation considerations (32).

To determine if inhibition of CDK signaling correlated with suppression of tumor growth, we examined Rb phosphorylation in tumor extracts from the Colo205 tumor xenograft study. Figure 5*C* shows that CDKi 277 dosed at 25 and 12.5 mg/kg inhibited or reduced, respectively, Rb phosphorylation in the Colo205 tumors when compared with the vehicle control. Overall, these results show that blocking CDK activity *in vivo* results in inhibition of tumor cell proliferation, inhibition of Rb phosphorylation, and suppression of tumor growth. In addition, by altering the dose and schedule of CDKi 277, we have shown that *in vivo* efficacy can be achieved with an acceptable therapeutic index.

Discussion

The increased expression of cyclins and activation of CDK complexes regulate the normal cell cycle, and their deregulation

in tumors contributes to tumor progression and poor patient survival rates. Although advancements have been made in developing CDK inhibitors with the appropriate properties for clinical development, many of the CDK inhibitors currently in clinical studies still have poor CDK selectivity, potency, and physical properties. There is a clear need for more potent and selective CDK inhibitors with the appropriate physical properties to determine if inhibition of CDK activity will prevent tumor progression in cancer patients.

Suppression of gene expression is one way to determine the functional role that specific protein plays in regulating the cell cycle. The CDK2 antisense and RNAi results are consistent with a recent report showing that inhibition of CDK2 expression had little effect on proliferation in several tumor-derived cell lines in cell culture (18); however, our results did indeed detect reproducible cell cycle effects (G_2-M increase) by coupling BrdUrd and DNA staining, whereas the recently reported data showed no cell cycle phenotype effect using CDK2 suppression methodologies. Our results show that CDK1 expression was required for osteosarcoma and breast tumor cell proliferation and that suppression of CDK1 decreased S phase while markedly increasing G_2-M. Techniques, such as RNAi, antisense, and overexpression of kinase-dead mutants, are commonly accepted methods for validation of drug targets. However, the cell phenotypes generally seen with small-molecule kinase inhibitors are consistently more dramatic than RNAi or kinase-dead mutants over a variety of kinase targets with distinct phenotypes. Perhaps a small-molecule inhibitor with a high affinity for the ATP-binding pocket of CDK2 may act more like a dominant-negative inhibitor and titrate out cyclins as well as proteins required for the catalytic activity of additional CDK complexes. In this respect, a CDK2 small-molecule inhibitor may have a different cellular phenotype compared with cells that have lost expression of CDK2. In support of this concept, reports have shown that in U2OS osteosarcoma cells suppression of CDK2 expression by antisense had no effect on cell cycle progression, whereas a dominant-negative CDK2 mutant increased the percentage of U2OS cells in G_1 and decreased the S-phase fraction (18, 34).

Our results show that CDKi 277 potently inhibited CDK2 and CDK1 enzyme activity with IC_{50}s of 4 and 8 nmol/L, respectively. In addition, when tested in a 24-hour proliferation assay, CDKi 277 had an average cellular IC_{50} of 0.243 nmol/L against a broad panel of tumor cell lines; furthermore, CDKi 277 was significantly

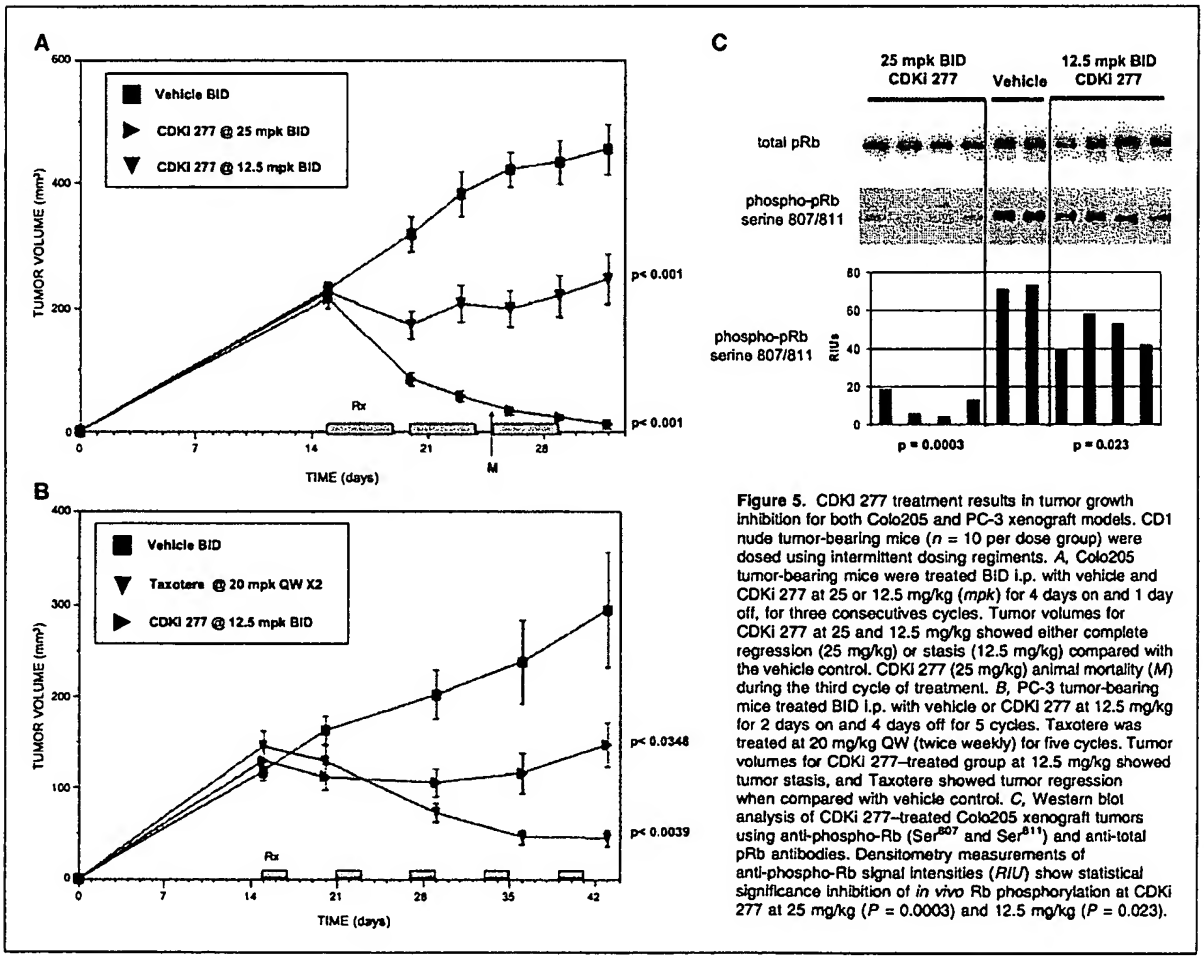


Figure 5. CDKI 277 treatment results in tumor growth inhibition for both Colo205 and PC-3 xenograft models. CD1 nude tumor-bearing mice (n = 10 per dose group) were dosed using intermittent dosing regiments. *A*, Colo205 tumor-bearing mice were treated BID i.p. with vehicle and CDKi 277 at 25 or 12.5 mg/kg (*mpk*) for 4 days on and 1 day off, for three consecutives cycles. Tumor volumes for CDKi 277 at 25 and 12.5 mg/kg showed either complete regression (25 mg/kg) or stasis (12.5 mg/kg) compared with the vehicle control. CDKI 277 (25 mg/kg) animal mortality (*M*) during the third cycle of treatment. *B*, PC-3 tumor-bearing mice treated BID i.p. with vehicle or CDKI 277 at 12.5 mg/kg for 2 days on and 4 days off for 5 cycles. Taxotere was treated at 20 mg/kg QW (twice weekly) for five cycles. Tumor volumes for CDKi 277–treated group at 12.5 mg/kg showed tumor stasis, and Taxotere showed tumor regression when compared with vehicle control. *C*, Western blot analysis of CDKi 277–treated Colo205 xenograft tumors using anti-phospho-Rb (Ser^{807} and Ser^{811}) and anti-total pRb antibodies. Densitometry measurements of anti-phospho-Rb signal intensities (*RIU*) show statistical significance inhibition of *in vivo* Rb phosphorylation at CDKi 277 at 25 mg/kg (P = 0.0003) and 12.5 mg/kg (P = 0.023).

more potent than Roscovitine, a CDK inhibitor currently in clinical trials. The proliferation assay correlated well with the IC_{50}s generated in the short-term Rb and PP-1α cellular phosphorylation assays. The ability of CDKi 277 to arrest cells in the G_1 and G_2 phase of the cell cycle and to induce tumor cell apoptosis is consistent with the reported role that CDK2 and CDK1 complexes play in regulating the cell cycle. The CDKi 277 apoptotic response was marked by an increase in the active forms of PARP and caspase-3, which also coincided with the phosphorylation of histone H2AX. CDKi 277 also inhibited Rb phosphorylation and E2F-mediated transcriptional activation, which is again consistent with the role that CDK complexes play in regulating Rb and its interaction with E2F complexes (data not shown). Our results show that the inhibition of Rb phosphorylation correlates with the ability of compounds to block tumor cell proliferation. However, CDKi 277 was equipotent in Rb-positive and Rb-negative tumor cells, suggesting that modulation of additional proximal CDK substrates was required for the cell cycle arrest. Additionally, CDKi 277 was equipotent on both p53-positive and p53-negative isogenic tumor cells and exhibited similar cell cycle arrest phenotypes. It is important to highlight that whereas our results with CDKi 277 were consistent with the biochemical and cellular phenotype expected for inhibition of CDK complexes (40), the possibility does exists that our CDK inhibitors may affect other signaling pathways.

CDKi 277 exhibited potent antitumor activity *in vivo*. By adjusting the dose and schedule for CDKi 277, we minimized toxicities and achieved tumor growth inhibition in both Colo205 and PC-3 tumor xenografts. In addition, the compound-treated mice showed a reduction in both Rb phosphorylation and BrdUrd incorporation in the tumor. Although CDKi 277 showed significant tumor inhibition as a monotherapy, in the clinic, CDK inhibitors will most likely be used in combination with either standard of care chemotherapeutics or with biologic-based therapies.

The precise mechanisms determining tumor cell fate after treatment with CDK inhibitors still remains elusive, gaining a better understanding of the survival and checkpoint adaptations will help elucidate the underlying factors regulating the phenotype response. Ultimately, CDK inhibitors with increased potency, better selectivity, and favorable drug-like properties have the greatest potential for clinical advancement.

Acknowledgments

Received 7/18/2005; revised 1/20/2006; accepted 2/9/2006.

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